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Metabolic inflammation in hepatic and vascular disorders

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Chapter 1

General introduction

The global burden of obesity

Although once considered a problem only in high-income countries, obesity is now also on the rise in low- and middle-income countries with prevalences increasing worldwide. In 2014, only a third of the world's population was considered to be of 'normal' weight (body mass index < 25 kg/m^2) and over 600 million adults were obese (body mass index ≥ 30 kg/m^2) [1]. These numbers reflect more than a doubling in the prevalence of obesity since 1980, and this rise is only projected to continue [1]. As obesity is associated with reduced life expectancy [2] and increased all-cause mortality [3] this obesity epidemic may represent one of the greatest threats to global human health.

The World Health Organization defines obesity as "abnormal or excessive fat accumulation that may impair health", and it is commonly classified as a body mass index ≥ 30 kg/m^2. Obesity is associated with several metabolic abnormalities such as abnormal blood cholesterol and triglyceride levels (dyslipidaemia) and elevated blood glucose and insulin levels (hyperglycaemia and hyperinsulinaemia) [4]. These features are thought to contribute to the development of obesity-associated metabolic diseases such as type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD), for which obesity is a major risk factor [4]. In addition to these metabolic derangements observed in obese individuals, inflammation has recently emerged as a driving force and potential unifying mechanism behind the pathogenesis of obesity-associated diseases.

Metabolic inflammation

Inflammation is a normal and essential defence mechanism that protects the host from infection and injury. It is the process by which an initial source of cellular injury – an invading pathogen or physical tissue damage – is eliminated in an orchestrated manner. An effective inflammatory response is self-limiting and initiates tissue repair processes, thereby promoting homeostasis at infected or damaged sites. Such regulated inflammatory responses are essential to maintain health.

Over the past few decades, it has become increasingly clear that inflammation can also be triggered by nutrients and metabolic surplus, an inflammatory state that has been coined 'metaflammation' (metabolically triggered inflammation) [5]. In contrast with classical inflammation, as described above, metabolic inflammation is not self-limiting and leads to a state of chronic low-grade inflammation that is considered to be a pathological feature of

obesity-associated diseases such as T2D, NAFLD and CVD [6]. This metabolic inflammatory response involves many components of the classical inflammatory response and is, at later stages, characterised by systemic increases in circulating inflammatory mediators such as cytokines (e.g. Tumour Necrosis Factor-alpha: TNF-α), bioactive lipids (e.g. palmitic acid, ceramides) and acute phase proteins (e.g. C-Reactive Protein: CRP and Serum Amyloid A: SAA) [6]. Obesity – inherently a state of metabolic excess – is characterised by metabolic inflammation: many studies have shown that tissues become inflamed and systemic inflammatory markers are increased in rodent models of obesity as well as in obese humans (e.g. [7-9]).

Mechanisms of metabolic inflammation

Although much remains unknown about the origin or mechanisms of metabolically triggered inflammation, the currently favoured paradigm is that metabolic inflammation is triggered when metabolic excess (surplus energy or macronutrients) is too big for designated metabolic cells (such as adipocytes or hepatocytes) to process. As a result, an inflammatory response is initiated in these specialised metabolic cells, either in direct response to high nutrient levels or in response to the cellular stress or damage that may be the result of metabolic overload, thus mediating the interface between metabolic input and inflammatory output [10]. Although metabolic organs such as the adipose tissue and the liver are likely to be the most susceptible to metabolic overload and inflammation, metabolic inflammation can also develop in other organs (e.g. kidney, brain and vasculature).

Sensing metabolic overload

It is now generally accepted that metabolic overload in metabolic cells results in activation of inflammatory pathways within these cells. Although the initial trigger of the metabolic inflammatory response remains unclear – and may differ according to diet and/or individual susceptibility – several molecules have been proposed to provide the link between metabolism and the immune system: acting as sensors of metabolic overload and activating inflammatory signalling pathways. These molecules belong to the classical pathogen-sensing pathways of the innate immune system, and are thought to be able to recognise excessive nutrient intake as a harmful, stress-related biological event [11].

The innate immune system – the first line of defence against infectious agents – relies on so-called pattern recognition receptors (PRRs) to monitor the extracellular space and intracellular

compartments for the presence of microbes, cell damage or other cellular stressors. These PRRs are able to detect pathogen-associated molecular patterns (PAMPs) as well as endogenous damage and stress signals through damage-associated molecular patterns (DAMPS) [12]. There are several classes of PRRs, of which the Toll-like receptors (TLRs) and the Nod-like receptors (NLRs) have emerged as potentially important regulators of metabolic inflammation [11].

TLRs are a family of membrane-bound pathogen sensing-receptors with at least twelve members in mammalian species, each of which appears to have a distinct function in innate immune recognition [12]. TLR2 and TLR4 are particularly interesting in the context of metabolic inflammation, as they are also broadly expressed by non-immune cells such as adipocytes, hepatocytes and myocytes [13-16] and can be activated by metabolic signals such as saturated fatty acids like palmitate [17-19], ceramides [20], and oxidised low-density lipoproteins (LDL) [21, 22]. Engagement of these TLRs results in the activation of intracellular pro-inflammatory signalling pathways, leading to activation of the transcriptional regulators NFκB and AP-1 [23]. Studies in rodent models of obesity have shown that genetic deletion of TLR2 or a loss-of-function mutation in TLR4 protects against high-fat-diet-induced inflammation [24, 25], thus providing evidence that these receptors may indeed be involved in sensing of metabolic inflammation.

NLRs are cytoplasmic PRRs that assemble into large multimeric protein complexes called inflammasomes. These inflammasomes consist of a sensor molecule (the NLR), the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and the effector protein caspase-1 [26]. The best characterised inflammasome is the NLR family pyrin domain-containing 3 (NLRP3) inflammasome, which can be activated by a variety of PAMPs and DAMPs. Like TLRs, NLRs are also expressed by metabolic cells [27, 28] and they can be activated by metabolic signals such as saturated fatty acids [29, 30] and crystallised cholesterol [31, 32]. In reaction to sensing of a PAMP or DAMP, the inflammasome protein complex is formed and pro-caspase-1 is autoproteolytically cleaved into caspase-1. Active caspase-1 then proteolytically activates the biologically inactive precursors of the cytokines IL-1β and IL-18 into their mature, pro-inflammatory counterparts [33]. These cytokines are released into the extracellular space, where they can bind to their respective receptors and activate pro-inflammatory signalling pathways in an autocrine or paracrine manner, contributing to local organ inflammation. Several studies have shown that genetic deletion of NLRP3-associated genes (i.e. ASC, NLRP3 or Caspase-1) in rodent models of obesity reduces diet-induced inflammation and metabolic disease development [30, 31, 34], suggesting that the

NLRP3 inflammasome contributes to metabolic inflammation.

Inflammatory response to metabolic overload

The activation of a PRR, either by a 'classical' DAMP or PAMP or by nutrient excess, leads to the activation of intracellular signalling pathways that converge on the activation of pro-inflammatory transcription factors to initiate a program of gene expression that enables the mounting of an inflammatory response. In the context of metabolic inflammation, the transcription factor NFκB has frequently been implicated as a key player [35-38]. NFκB is considered the master regulator of inflammatory responses and although it was initially characterised in immune cells, it is now known to be expressed and capable of activation in most cell types [36]. Under resting conditions, NFκB is sequestered in the cytosol by inhibitor of κB (IκB), which prevents its nuclear localisation and transcriptional function. In response to stimulation of PRRs – e.g. by metabolic overload – intracellular signalling pathways converge on the activation of inhibitor of κB kinase (IKK), which phosphorylates IκB and thereby promotes its degradation. This releases NFκB, allowing for its translocation to the nucleus where it promotes transcription of its target genes [39]. Since the NFκB target genes include a wide array of pro-inflammatory cytokines, chemokines and adhesion molecules, activation of NFκB by nutritional overload contributes to immune cell recruitment and amplifies and sustains an inflammatory reaction that ultimately results in organ dysfunction and thereby metabolic disease development [37].

This recruitment of immune cells into tissues is a common component of metabolic diseases. In a classical inflammatory response, the first immune cells that are recruited to an inflammatory site are the neutrophils (polymorphonuclear leukocytes), which mediate the earliest phase of the innate immune response [40]. They respond to chemoattractants that are produced by resident tissue macrophages in response to inflammatory or cellular-damage signals released from parenchymal cells [41]. As the body's first line of defence during acute inflammatory responses, neutrophils are highly efficient, short-lived effector cells that carry a multitude of weapons that are either primarily directed against a wide range of pathogens, or serve as signal molecules for the recruitment and activation of secondary immune cells [42]. The brute force and indiscriminate nature of the potent arsenal of antimicrobial weaponry (e.g. proteases and reactive oxygen species) carried by neutrophils makes them a potential cause of extensive tissue damage, which is thought to contribute to their detrimental role in metabolic disease development [43, 44]. Monocyte-derived macrophages typically follow soon after the neutrophils, but survive much longer at sites of inflammation [40]. The primary function of

these phagocytes is to identify, ingest and destroy pathogens, and they play an important role in the clearance of apoptotic cells and cellular debris. Unlike neutrophils, macrophages are not terminally differentiated and they show a remarkable phenotypic plasticity in response to their environment. During obesity, the macrophage population differs from that of lean subjects not only in number and tissue localisation, but also in inflammatory phenotype [45]. Although neutrophils and macrophages have an essential role in the protection of the host against invading pathogens, their improper recruitment and activation during metabolic inflammation can contribute to amplification and propagation of the initial inflammatory signal, thereby driving organ dysfunction and disease progression.

Defective resolution in metabolic inflammation

While the ideal inflammatory response is self-limiting, metabolic inflammation is chronic in nature. The self-limiting capacity of an inflammatory response relies on the timely initiation of inflammation resolution, which allows for the repair of injured tissues and enables a return to homeostasis. Although inflammation resolution was long thought to be a passive process, it is now recognised to be an active programmed response that is orchestrated by a family of lipid mediators that are derived from essential fatty acids: the specialised pro-resolving mediators (SPMs) [46]. These SPMs, namely resolvins, protectins, and maresins, act as agonists to actively dismantle the inflammatory response, limit further leukocyte recruitment, promote efferocytosis (the uptake and clearance of apoptotic cells, cellular debris and microorganisms by macrophages) and initiate tissue repair mechanisms [47, 48]. Dysfunctional resolution is thought to underlie the aetiology of chronic inflammatory diseases [49], and it has been proposed that defective resolution of inflammation may be pivotal to the sustained inflammatory state that drives metabolic disease development [50]. When resolution of inflammation is defective there is no appropriate termination and clearance of phagocytes, and the resulting sustained presence of activated leukocytes within tissues is associated with collateral tissue damage, amplification of the inflammatory response and persistence of tissue inflammation, ultimately leading to organ dysfunction [50].

Organ dysfunction during metabolic disease development

Adipose tissue dysfunction

The adipose tissue is the primary site of energy storage in the body. When energy intake exceeds energy expenditure (i.e. dietary excess) energy is stored in the adipose tissue in the form of triglycerides, leading to expansion of adipose tissue depots. This adipose tissue

expansion is the result of both adipocyte hyperplasia (increase in cell number) and adipocyte hypertrophy (increase in cell size). In addition to the fact that excess lipids or intermediates of lipid metabolism could directly trigger an inflammatory response by engaging with PRRs as discussed above, it is thought that cellular stress, local hypoxia, intracellular damage (e.g. to membranes or organelles) and eventually necrosis may occur when an adipocyte has reached the limit of its expansion, thus providing additional triggers for inflammation [51, 52]. These inflammatory signals lead to expression of chemotactic signals by adipocytes, resulting in infiltration of immune cells into the adipose tissue [52-54] where they characteristically arrange around necrotic adipocytes in so-called crown-like structures [55]. Secretion of pro-inflammatory cytokines such as IL-1β and TNF-α [9, 56] by these infiltrating immune cells further propagates the inflammatory response. These pro-inflammatory cytokines also stimulate lipolysis [57] and contribute to insulin resistance (discussed below), thus promoting dysfunction of adipose tissue. In addition to these effects within the adipose tissue, it is thought that spillover of inflammatory and metabolic signals from dysfunctional adipose tissue into the circulation may drive the progression of organ dysfunction and metabolic disease in other organs such as the liver and the vasculature [58]. Adipose tissue dysfunction and inflammation is thus widely considered to play a crucial role in obesity-associated metabolic inflammation and disease development [5, 6, 58-60].

Insulin resistance and type 2 diabetes

It is now generally accepted that (metabolic) inflammation can interfere with insulin signalling pathways in various tissues, leading to reduced insulin sensitivity and ultimately to development of insulin resistance and type 2 diabetes (T2D) [61]. Pro-inflammatory cytokines such as TNF-α and IL-1β are thought to interfere with insulin signalling downstream of the insulin receptor, thus making insulin target cells less sensitive to its effects [56, 62, 63]. This means that glucose is not properly cleared from the circulation, leading to increased levels of blood glucose (hyperglycaemia). Of note, recent observations indicate that this interplay between inflammatory cascades and insulin signalling is more complex than generally assumed [64] and show that aggravation of hepatic inflammation does not necessarily worsen insulin resistance [65]. Hyperglycaemia resulting from reduced glucose clearance is further exacerbated by inappropriately increased glucose release from the liver, resulting from the inability of insulin to suppress hepatic gluconeogenesis [66]. In the early stages of insulin resistance, the pancreatic beta cells increase insulin secretion in a compensatory attempt to lower circulating glucose levels. However, this compensation is lost in individuals with overt

type 2 diabetes due to beta cell dysfunction and depletion [67], causing glucose levels to rise uncontrollably.

There is a well-established relationship between increased adiposity – particularly in abdominal visceral fat depots – and reduced whole-body insulin sensitivity in obese subjects [68, 69]. Since the adipose tissue only removes a small fraction of plasma glucose, the expanded adipose tissue depots must also affect insulin sensitivity in other tissues such as skeletal muscle and liver, together resulting in reduced whole-body insulin sensitivity [66]. Rather than developing in parallel in different tissues, different organs may become insulin resistant at different stages of metabolic overload.

Non-alcoholic fatty liver disease

The liver is the primary organ for energy homeostasis as it plays a major role lipid and carbohydrate metabolism. In the fed state, dietary lipids (triglycerides and cholesterol) are absorbed and packed into triglyceride-rich chylomicrons in the intestine, which are transported to the blood through the lymphatic system. At peripheral sites, chylomicron-derived triglycerides are hydrolysed to allow uptake of free fatty acids for energy supply (skeletal muscle and heart) or storage (adipose tissue). The resulting chylomicron remnants (now triglyceride-depleted and cholesterol-enriched) are taken up by the liver where they are metabolised or stored. In the fasted state, the liver ensures the supply of triglycerides to peripheral organs through secretion of very low density lipoproteins (VLDL). The assembly of these triglyceride-rich particles is highly dependent of the availability of triglycerides within the hepatocyte, which may originate from: previously accumulated triglycerides within the hepatocyte; free fatty acids released from adipose tissue, re-esterified in the liver; or triglycerides synthesised in the liver from de novo synthesised free fatty acids. Similarly to chylomicrons, triglycerides from VLDL are hydrolysed at peripheral sites and the resulting VLDL remnants (cholesterol-enriched low-density lipoproteins: LDL) are cleared by the liver. Carbohydrates enter the circulation as glucose, after digestion of complex carbohydrates following a meal. Excess glucose is either converted into glycogen (the primary hepatic storage form of carbohydrates), or used as substrate for de novo lipogenesis. In the fasted state, the liver ensures adequate plasma glucose levels by releasing glucose from stored glycogen (glycogenolysis) or de novo synthesised glucose (gluconeogenesis) [70]. This central role of the liver in the regulation of metabolic homeostasis may make it particularly vulnerable to the effects of metabolic overload.

The hepatic manifestation of metabolic overload and inflammation is non-alcoholic fatty liver

disease (NAFLD). This chronic liver disease encompasses a spectrum of liver pathologies that ranges from the clinically benign accumulation of lipids (simple steatosis) to the more progressive non-alcoholic steatohepatitis (NASH) which is characterised by hepatic inflammation and fibrosis in addition to hepatic steatosis [71]. The intrahepatocellular accumulation of lipids (mainly triglycerides, but also free fatty acids and cholesterol) in NAFLD is thought to be the result of increased dietary influx, peripheral insulin resistance (which increases free fatty acid flux from the adipose tissue) and increased hepatic de novo lipogenesis [72]. While triglycerides are considered a relatively "safe" storage form of lipids in the liver, it is the build-up of other lipid species such as free fatty acids, diglycerides, ceramides, and free cholesterol that is thought to provide a direct trigger of hepatic inflammation [73]. In addition, circulating factors released from dysfunctional adipose tissue have also been proposed to exert pro-inflammatory effects in the liver [74] and may thereby contribute to the progression of NAFLD. Continued hepatic inflammation then drives the development of hepatic fibrosis, through activation of hepatic stellate cells by pro-inflammatory mediators such as TNF-α and TGF-β, which stimulates their production of collagen [75].

Atherosclerosis

As the underlying cause of myocardial infarction, stroke and sudden cardiac death, atherosclerosis is the major underlying pathology of cardiovascular disease (CVD) [76]. Although atherosclerosis was initially considered a passive accumulation of cholesterol debris on the arterial wall, atherosclerosis is now recognised to be a chronic non-resolving inflammatory disease [77]. It is triggered by an interplay between endothelial dysfunction and subendothelial (intimal) low-density lipoprotein (LDL) retention, and occurs in medium-sized arteries at regions of disturbed blood flow (i.e. at bends or branch points) [78]. Various modifications (such as oxidation) of these retained lipoproteins are thought to mimic PAMPs or DAMPs, thereby triggering an inflammatory response. This leads to activation of endothelial cells and their expression of adhesion molecules. Together with the release of chemokines this attracts leukocytes (primarily monocyte-derived macrophages, but also neutrophils and other immune cells) that subsequently infiltrate the vascular wall and contribute to amplification and perpetuation of the inflammatory response [78].

Targets for intervention in metabolic inflammation

Obviously, lifestyle interventions that focus on reduction of metabolic overload (e.g. restriction of food intake) may at least partly prevent metabolic inflammation and development of metabolic diseases. However, such lifestyle changes are difficult to achieve in the long run,

which urges the development of therapeutics to combat these disorders. The major challenge in the field of metabolic disease is to prevent, dampen, or resolve the metabolic inflammatory response that is the driving force of metabolic disease development (both initiation and progression). This may be achievable by interventions targeted to: A) prevent metabolic overload by changing the macronutrient composition of the diet to prevent nutrient excess, B) inhibit sensing of metabolic overload (i.e. at the level of translation of metabolic to inflammatory signal), C) reduce amplification of the early metabolic inflammatory response, D) stimulate resolution of inflammation to prevent development of chronic inflammatory responses. Herein, we investigated several routes of intervention in metabolic inflammation using both pharmaceutical and nutraceutical approaches, to assess their value in the attenuation of metabolic disease development focusing on NAFLD and atherosclerosis as main disease endpoints.

Aim and outline of this thesis

Although it is considered to be of great importance in the development of obesity-related diseases such as NAFLD and atherosclerosis, much remains unknown about the origin and mechanisms of metabolic overload and inflammation. The aim of this thesis was to further our understanding of metabolic inflammation and investigate the effects of interventions targeted to different aspects of metabolic inflammation on the development of disease endpoints. First, we aimed to unravel the contribution of different organs (liver and adipose tissue) to the development of metabolic inflammation and disease (**chapter 2**). In this chapter we explored the sequence of inflammatory events in adipose tissue and liver during metabolic overload in a longitudinal study in high-fat diet (HFD)-fed C57BL/6J mice, and investigated the contribution of adipose tissue- and liver inflammation to systemic metabolic inflammation and insulin resistance. We then sought evidence for a causal role of inflamed adipose tissue in the development of NAFLD in **chapter 3**. Using HFD-fed C57BL/6J mice, we studied the development of metabolic inflammation in several (visceral) adipose tissue depots and investigated whether surgical removal of inflamed white adipose tissue may reduce hepatic inflammation and thereby attenuate progression of NAFLD to NASH. Next, we studied interventions targeted to a specific factor in metabolic inflammation and investigated whether these may affect metabolic disease development. In **chapter 4** this was the NLRP3 inflammasome, i.e. the sensing of metabolic overload, in which we intervened using a caspase-1 inhibitor. For this we treated HFD-fed LDLr-/-.Leiden mice with a caspase-1 inhibitor in a

therapeutic study protocol, and investigated its effects on adipose tissue inflammation, insulin resistance and hepatic steatosis, inflammation and fibrosis. We then evaluated distinct nutritional strategies to prevent metabolic inflammation and disease, either by changing the macronutrient composition of the diet to reduce metabolic overload, or by adding potentially anti-inflammatory nutrients to the diet. In **chapter 5** we investigated whether replacement of dietary saturated fat with pumpkin seed oil (rich in unsaturated fat) would reduce NAFLD and atherosclerosis development and questioned whether potentially bioactive phytochemicals present only in unrefined pumpkin seed oil may have additional beneficial effects on top of those of the refined oil. For this we fed ApoE*3Leiden mice a cholesterol-containing Western-type diet, in which we replaced part of the saturated fat in this diet (cocoa butter) with either refined or virgin pumpkin seed oil and studied the effects of these interventions on NAFLD and atherosclerosis development. To gain more insight into the potential health effects of phytochemicals, we conducted two studies which either focused on metabolic inflammation in liver during NASH development or metabolic inflammation in vasculature during atherosclerosis development. In **chapter 6** we evaluated the effects of a mixture of phytochemicals (bilberry extract) on the development of NASH and hepatic fibrosis in Western-type diet fed ApoE*3Leiden mice, specifically focusing on the role of cholesterol as an inducer of hepatic inflammation. In **chapter 7** we studied the potential of the phytochemical (-)-epicatechin to prevent metabolic inflammation and atherosclerosis development. We studied the potential anti-inflammatory and anti-atherosclerotic effects of this phytochemical in Western-type diet fed ApoE*3Leiden mice and performed additional mechanistic studies of its anti-inflammatory potential in diet-induced inflammation in human-CRP transgenic mice and NFκB-luciferase reporter mice. Finally, in **chapter 8**, we evaluated a conceptually different strategy to attenuate metabolic inflammation, namely targeting resolution of inflammation using an SPM (Resolvin E1) with particular emphasis on vascular inflammation and atherosclerosis development, using a therapeutic study protocol in Western-type diet fed ApoE*3Leiden mice. The results obtained in these studies and their implications are discussed in **chapter 9**.

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